

# Lessons learnt from many years of experience using anti-D in humans for prevention of RhD immunization and haemolytic disease of the fetus and newborn

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## Summary

For 40 years prophylactic anti-D has been given to D-negative women after parturition to prevent haemolytic disease of the fetus and newborn. Monoclonal or recombinant anti-D may provide alternatives to the current plasma-derived polyclonal IgG anti-D, although none of them have yet proved as effective in phase 1 clinical trials. The variation in efficacy of the antibodies may have been influenced by heterogeneity in glycosylation of anti-D produced from different cell lines. Some aspects of the conduct of the human studies, most notably the use of low doses of anti-D and target D positive red cells *in vivo*, may aid the design of the clinical development of other immunomodulatory drugs in order to minimize adverse effects.

**Keywords:** anti-D, clinical trials, monoclonal antibody, red cell clearance, RhD haemolytic disease

The recent editorial by Dayan and Wraith [1] in this journal highlighted the challenges for developing new immunotherapies after the disastrous trial of TGN1412. This overview presents some of the knowledge gained from many clinical trials of anti-D that may be relevant for translational immunology.

Prevention of hydrops fetalis or Rhesus haemolytic disease of the fetus and newborn (HDFN) by prophylactic anti-D is the most successful clinical application of antibody-mediated immunosuppression. HDFN occurs after a D-negative woman becomes immunized to fetal D-positive red blood cells following fetomaternal haemorrhage (FMH); the IgG anti-D produced is transferred across the placenta causing destruction of fetal red cells by splenic macrophages. In the 1940s, when the cause of this disease was first recognized, 1% of babies were born with HDFN and 40% of them died [2]. Anti-D is the commonest antibody implicated. The RhD polypeptide on red cells is the most immunogenic of the blood group antigens as it is absent from cells of D-negative individuals who lack the *RHD* gene [3].

HDFN is now rare, partly due to improved fetal and neonatal care but mainly because of prevention of primary immunization of susceptible D-negative women by prophylactic IgG anti-D [1]. Since 1968, after successful clinical trials in the UK [4] and USA [5], anti-D has been given to 10% of all women postnatally, resulting in a reduction in the incidence of the disease of about 95%. Fewer than 30 perinatal deaths per year are now caused by HDFN. In 2002, the National Institute of Health and Clinical Excellence (NICE)

recommended routine antenatal anti-D prophylaxis be given to all D-negative women, in addition to postnatal prophylaxis, to reduce the immunization rate still further. Intravenous (IV) anti-D is also now used therapeutically to treat some D-positive patients with immune thrombocytopenic purpura (ITP) [6]. Thus the demand for anti-D is increasing.

Anti-D immunoglobulin is prepared from pooled hyperimmune human plasma. For many years, issues over virological safety and, more recently, variant Creutzfeldt Jacob disease (vCJD) have stimulated the search for alternative supplies. In the UK, plasma for fractionation is now sourced from North American donors because of concerns over UK donors being latent carriers of vCJD. With the aim of replacing polyclonal anti-D prepared from human plasma with biotech versions for both diagnostic and clinical use, hundreds of anti-D monoclonal (mAb) or recombinant (rAb) antibodies have been produced. They are all derived from human immunoglobulin genes or B cells because mice do not recognize the RhD antigen. Various expression systems have been used, including human B-cell lines, Chinese hamster ovary (CHO) cells, mouse-human heterohybridomas and rat myelomas.

Although the exact mechanism of suppression of D immunization by administration of passive IgG anti-D remains to be elucidated [7], it is known that D-positive red cells are rapidly cleared to the spleen by macrophages via IgG Fc receptor (FcγR) interactions and rendered non-immunogenic. Indeed, to ensure prophylactic anti-D is likely to be effective at preventing D immunization to a large FMH,

women are tested 2–3 days later to check that fetal cells are cleared from the circulation [8]. Otherwise, allogeneic red cells have a long survival after FMH or transfusion. Alloimmune responses are slow to develop, typically 5–15 weeks for anti-D [9]. This is probably due to their lack of danger signals (from foreign molecules) so that they are not recognized by the immune system until they become senescent, which then stimulates their phagocytosis via phosphatidyl serine receptors [10]. Without RhD prophylaxis, approximately 17% of D-negative women become immunized after pregnancy with a D-positive fetus [11]. This incidence is lower than for deliberately immunized normal subjects (up to approximately 85% respond [9]) because for most women the volumes of FMH are too small for the red cells to be immunogenic [12]. Pregnant women can make robust alloimmune responses whilst tolerating their semi-allogeneic fetus.

Over the last 20 years, 19 anti-D mAbs and rAbs were tested in 15 first-in-man studies. These were recently reviewed [13] and are summarized here. No serious adverse effects occurred. *In vitro* biological assays of FcγR-mediated phagocytosis and haemolysis using human effector cells are well established [14] and were used for screening. The clinical trials assessed the ability of the antibodies to remove small volumes (less than 1%) of D-positive red cells from the circulation and in some studies the ability of the anti-D to prevent D immunization was also then determined. Many of the mAbs and rAbs were directly compared with polyclonal anti-D.

Great heterogeneity in antibody efficacy was observed (Table 1). Two mAbs derived from human B-lymphoblastoid cell lines, BRAD-3 and BRAD-5, mediated rapid red cell clearance and prevented D immunization almost as effectively as polyclonal anti-D, although a three- to fourfold higher dose was used [15,16]. The plasma half lives of BRAD-3 and BRAD-5 were normal but bioavailability was half that of polyclonal anti-D [17]. When these antibodies were expressed as rAbs in CHO cells, clearance of autologous D-positive red cells was slower than the original mAbs [18]. A large study using another CHO-derived anti-D rAb, MonoRho, gave disappointing results, red cell clearance being extremely variable, usually very slow and with no correlation to the dose of anti-D [19]. The very low bioavailability of MonoRho [20] might partly explain this. The subjects did not, however, produce anti-D, although they were not given challenge injections of D-positive red cells to determine their D immunization status [19]. MAbs produced by murine myeloma cell lines (as mouse-human heterohybridomas) also showed great variability in red cell clearance [21–23] but unexpectedly, over half the recipients rapidly became D immunized [22,23], twice as many as would occur with red cells alone [9]. Thus these anti-Ds had an adjuvant effect, enhancing the immune response to D-positive red cells instead of preventing it as intended. Later studies using anti-D rAbs produced by rat myeloma cells

**Table 1.** Summary of data from clinical trials of polyclonal anti-D and anti-D mAbs and rAbs.

Cell used for expression	Clone	Rate of red cell clearance (zero to rapid: – to +++++)	Effect on RhD immunization	Reference
Human B	Polyclonal	Rapid, little variation among subjects (++++)	Prevented	[15,19,22,25]
Human B lymphoblastoid cell line	BRAD-3, BRAD-5 (mAbs)	Rapid, little variation among subjects, but 3x higher dose than polyclonal anti-D used (++++)	Prevented in 90% of subjects	[15,16]
CHO	BRAD-3+BRAD-5 (blend) (mAbs)	Slower than blend of mAbs BRAD-3+BRAD-5 (++)	(Not done)	[18]
CHO	BRAD-3+BRAD-5 (blend) (rAbs)	Very variable between subjects (+ to +++)	Prevented?	[19]
Mouse myeloma	G7, G12, G17, G48 (mAbs)	Very variable between subjects (– to +++)	Increased, rapid anti-D response	[22]
Mouse myeloma	AD1+AD3 (mAbs)	Slow and variable (+)	Increased, rapid anti-D response	[23]
Mouse myeloma	FOG-1 (mAb)	Rather slow and variable (+ to ++)	(Not done)	[21]
Rat myeloma	FOG-1 & mutants (rAbs)	Extremely rapid, even in absence of FcγR binding (+++++)	(Not done)	[24]
Rat myeloma	R297 (rAb)	Extremely rapid (+++++)	(Not done)	[25]

All clinical trials varied, making direct comparison difficult.

Clearance studies were performed in D-positive (autologous) or D-negative subjects, with red cells being injected before or after anti-D. Volumes of red cells ranged from 0.5 to 15 ml. Doses of anti-D differed (between 100 and 1800 µg) and anti-D was administered either on pre-coated cells or injected i.v. or i.m. Clearance studies were performed for between 1 h and 7 days, with varied timings of sample collection. One to 94 subjects were enrolled. In some studies, clearance rates were calculated.

Detection of anti-D responses was determined either in samples taken every 2 or 4 weeks or in a single 3- or 6-month sample; red cell challenge injections (secondary immunization) were given in only two studies [15,16].

**Table 2.** Design of phase 1 clinical trials of anti-D.

Tests and analyses	Details and comments	Points to consider for future development
Preclinical animal models <i>In vitro</i> bioassays	Not suitable for anti-D as the RhD antigen is restricted to humans Selection of candidate anti-D mAbs and rAbs was made using well-established tests of FcγR functional activity. Extensive collaboration in developing these bioassays was undertaken in four international workshops [14]	Analysis of the trial data suggests it would be beneficial to develop additional bioassays to study interactions of antibodies (or antibody coated cells) with components of the innate immune system for both anti-D and for other drugs targeting the immune system
Dose	The doses of both anti-D and target D-positive red cells were low in both the original clinical experiments conducted over 40 years ago and in the recent trials. The aim was for the anti-D to clear red cells acquired by FMH	In the TGN1412 phase 1 study, the amount of antibody administered was far too high and all T cells were targets, leading to the cytokine storm. The dose of antibody and antigen could be minimized if a sample of cells were coated with the test antibody <i>in vitro</i> , washed and injected
Tracers	The use of <sup>51</sup> chromium to label target red cells <i>ex vivo</i> enabled highly sensitive determination of their survival, tissue distribution and haemolysis <i>in vivo</i>	<sup>51</sup> Chromium or other radionuclides or fluorochromes should be considered in future studies of immunotherapeutics to both minimize doses and enhance the quality of information from trials
Dosing interval	In an anti-D study conducted before economic considerations were important, the dosing interval between subjects was 1 week, allowing time to monitor subjects for adverse effects [15]	Time periods between administering trial substances to volunteers should be considerably longer than the few minutes between subjects in the TGN1412 trial
Antigen-negative subjects	A possibly unique advantage of anti-D is that accurate determination of its bioavailability, pharmacokinetics and half life is possible because D-negative individuals lack the antigen	
Glycosylation of IgG Information sharing	It is likely that non-human glycosylation of anti-D rendered it pro- rather than anti-inflammatory. Biological effects were observed even at very low doses Combining results from all the clinical trials was more informative for analysing the immunological activity of the anti-D antibodies than individual studies alone [13]	The glycosylation of TGN1412 may have affected its activity <i>in vivo</i>  Publication advances knowledge

showed they promoted extremely rapid clearance of autologous red cells, faster than polyclonal anti-D [24,25]. The effect of altering the cell line expressing FOG-1 from mouse to rat was striking, changing the clearance from very slow and incomplete to very fast [21,24]. In the latter study this was associated with haemolysis, some clearance to the liver and febrile reactions [24]. These responses do not occur after prophylaxis with polyclonal anti-D. Unexpectedly, mutants of FOG-1 rAb, that lacked FcγR interactions *in vitro*, also mediated rapid red cell clearance, although normal survival had been expected [24]. These IgG anti-Ds must have bound to receptors other than IgG FcγR.

Many of the anti-D mAbs and rAbs did not behave like polyclonal anti-D. None were quite as effective and some from rodent cell lines even resulted in undesirable immune responses. The *in vivo* responses were determined mainly by the species of cell line producing the antibodies and not by the protein sequences. The cause of these unexpected and possibly harmful reactions may be because the IgG anti-D produced from animal cells interacted with components of the innate immune system. The most likely explanation is variation in their oligosaccharide composition.

The type of glycosylation of IgG depends on the cell in which it is produced and is species-specific. Structures such as *N*-glycolyl neuraminic acid and high mannose oligosaccharides on rodent IgG [26,27] may be recognized as foreign by innate immune pattern recognition receptors (PRRs) [28] with subsequent pro-inflammatory responses. PRRs include cellular asialoglycoprotein and mannose receptors. After binding anti-D on D-positive red cells, they might have stimulated antibody responses to the D antigen. In plasma, mannan binding lectin may cause complement mediated haemolysis when bound to mannose residues of anti-D on a red cell. Endogenous IgG antibodies [29] recognizing galactose-α1,3-galactose on murine IgG [30] could bind anti-D expressing this oligosaccharide. Recently, endogenous IgE anti-galactose-α1,3-galactose has caused hypersensitivity reactions in some patients given cetuximab (a cancer immunotherapeutic) produced in mouse myeloma SP2/0 cells [31]. Lack of sialic acid on many anti-D mAbs and rAbs would render the IgG pro-inflammatory upon binding FcγR [32].

Polyclonal anti-D can be either beneficial or lethal in different clinical settings. The dose of target red cells is a major factor. Clearance of small volumes of red cells, as in FMH, is a 'silent', non-inflammatory, non-haemolytic process. If, however, a D-positive individual receives large doses of anti-D, as in a fetus suffering RhD haemolytic disease or, rarely, ITP patients treated with IV anti-D, severe haemolysis can occur. In these occasionally fatal cases, additional symptoms are usually hydrops (oedema) in HDFN [2] and acute haemoglobinaemia, haemoglobinuria and disseminated intravascular coagulation in ITP patients [33]. Most cases of acute haemolysis were considered to be due to robust extravascular haemolysis (macrophage mediated) rather

than intravascular haemolysis [34]. Even in the absence of such serious adverse events, ITP patients not uncommonly experience fever and chills after infusion of IV anti-D [34], indicative of inflammatory reactions. Thus if ITP patients were treated with pro-inflammatory anti-D mAbs or rAbs instead of the current polyclonal anti-D, a complex series of unintended and potentially dangerous reactions might follow.

In the aftermath of the TGN1412 phase 1 study, some aspects of the clinical trials of anti-D may be helpful for the future development and regulation of first-in-man studies of other immunotherapeutics [1], especially those targeting cells in blood (Table 2). Choice of starting dose is particularly important. Low doses of red cells and anti-D were used in all the human work [13]. In some studies, for example for BRAD-3 [21], autologous red cells (0.5 ml) were coated with anti-D *ex vivo* then washed and injected, thus minimizing the dose of antibody and mitigating the possibility of adverse effects. Clearance of such small volumes of red cells could be accurately followed when they were isotopically labelled. When this antibody proved safe and effective, anti-D and red cells were then injected separately [15,16] to simulate more closely the clinical situation. Evidence of inflammatory reactions with low doses of anti-D and red cells [24] should ensure caution in scale-up studies.

In conclusion, the extensive data from human studies of anti-D mAbs and rAbs suggests that their glycosylation might have had a powerful effect in modulating their *in vivo* activities. Some rAbs increased rather than decreased the incidence of D immunization, one caused harmful haemolytic reactions and one had an extremely low bioavailability. These effects were not predicted from the *in vitro* studies performed. In future, when developing recombinant glycoproteins for human therapy, consideration should be given to the possibility that interactions between non-human oligosaccharides and cells or molecules other than the intended ligand may occur.

## References

- Dayan CM, Wraith DC. Preparing for first-in-man studies: the challenges for translational immunology post-TGN1412. *Clin Exp Immunol* 2008; **151**:231–4.
- Bowman J. Thirty-five years of Rh prophylaxis. *Transfusion* 2003; **43**:1661–6.
- Colin Y, Chérif-Zahar B, Van Kim L *et al*. Genetic basis of the RhD-positive and RhD-negative blood group polymorphism as determined by Southern analysis. *Blood* 1991; **78**:2747–52.
- Finn R, Clarke CA, Donohue WTA *et al*. Experimental studies on the prevention of Rh haemolytic disease. *BMJ* 1961; **1**:1486–90.
- Freda VJ, Gorman JG, Pollack W. Successful prevention of experimental Rh sensitization in man with an anti-Rh gamma2-globulin antibody preparation. A preliminary report. *Transfusion* 1964; **4**:26–32.
- Cooper N, Heddle NM, de Haas M *et al*. Intravenous (IV) anti-D and IV immunoglobulin achieve acute platelet increases by differ-

- ent mechanisms: modulation of cytokine and platelet responses to IV anti-D by FcγRIIa and FcγRIIIa polymorphisms. *Br J Haematol* 2004; **124**:511–18.
- 7 Kumpel BM, Elson CJ. Mechanism of anti-D-mediated immune suppression – a paradox awaiting resolution? *Trends Immunol* 2001; **22**:26–31.
  - 8 Guidelines. The estimation of fetomaternal haemorrhage. BCSH Blood Transfusion and Haematology Task Forces. *Transfus Med* 1999; **9**:87–92.
  - 9 Gunson HH, Stratton F, Phillips PK. The primary Rh<sub>0</sub>(D) response in male volunteers. *Br J Haematol* 1976; **32**:317–29.
  - 10 Kumpel BM. On the immunologic basis of Rh immune globulin (anti-D) prophylaxis. *Transfusion* 2006; **46**:1652–6.
  - 11 Woodrow JC. Rh immunization and its prevention. *Ser Haematol* 1970; **3**:1–151.
  - 12 Bowman JM, Pollock JM, Penston LE. Fetomaternal transplacental hemorrhage during pregnancy and after delivery. *Vox Sang* 1986; **51**:117–21.
  - 13 Kumpel BM. Efficacy of RhD monoclonal antibodies in clinical trials as replacement therapy for prophylactic anti-D immunoglobulin: more questions than answers. *Vox Sang* 2007; **93**: 99–111.
  - 14 Kumpel BM, Béliard R, Brossard R *et al.* Section 1C: assessment of the functional activity and IgG Fc receptor utilisation of 64 IgG Rh monoclonal antibodies. Coordinator's report. *Transfus Clin Biol* 2002; **9**:45–53.
  - 15 Kumpel BM, Goodrick MJ, Pamphilon DH *et al.* Human Rh D monoclonal antibodies (BRAD-3 and BRAD-5) cause accelerated clearance of Rh D<sup>+</sup> red blood cells and suppression of Rh D immunization in Rh D<sup>+</sup> volunteers. *Blood* 1995; **86**:1701–9.
  - 16 Smith NA, Ala FA, Lee D *et al.* A multi-centre trial of monoclonal anti-D in the prevention of Rh-immunisation of RhD- male volunteers by RhD<sup>+</sup> red cells. *Transfus Med* 2000; **10** (Suppl. 1):8.
  - 17 Goodrick J, Kumpel B, Pamphilon D *et al.* Plasma half-lives and bioavailability of human monoclonal Rh D antibodies BRAD-3 and BRAD-5 following intramuscular injection into Rh D-negative volunteers. *Clin Exp Immunol* 1994; **98**:17–20.
  - 18 Chapman GE, Ballinger JR, Norton MJ *et al.* The clearance kinetics of autologous RhD-positive erythrocytes coated ex vivo with novel recombinant and monoclonal anti-D antibodies. *Clin Exp Immunol* 2007; **150**:30–41.
  - 19 Miescher S, Spycher MO, Amstutz H *et al.* A single recombinant anti-RhD IgG prevents Rhesus D immunization: association of RhD positive red blood cell clearance rate with polymorphisms in the FcγRIIa and IIIa genes. *Blood* 2004; **103**:4028–35.
  - 20 Bichler J, Spycher MO, Amstutz H-P, Andresen I, Gaede K, Miescher S. Pharmacokinetics and safety of recombinant anti-RhD in healthy RhD-negative male volunteers. *Transfus Med* 2004; **14**:165–71.
  - 21 Thomson A, Contreras M, Gorick B *et al.* Clearance of Rh D-positive red cells with monoclonal anti-D. *Lancet* 1990; **336**:1147–50.
  - 22 Olovnikova NI, Belkina EV, Drize NI *et al.* Fast clearance of Rhesus positive erythrocytes by monoclonal anti-Rhesus antibodies – insufficient condition for effective prevention of Rhesus sensitization. *J Exp Biol Med* 2000; **129**:77–81 (in Russian).
  - 23 Béliard R. Monoclonal antibodies to prevent alloimmunization: lessons from clinical trials. *Transfus Clin Biol* 2006; **13**:58–64.
  - 24 Armour KL, Parry-Jones DR, Beharry N *et al.* Intravascular survival of red cells coated with a mutated human anti-D antibody engineered to lack destructive activity. *Blood* 2006; **107**:2619–26.
  - 25 Béliard R, Waegemans T, Notelet D *et al.* A human anti-D monoclonal antibody selected for enhanced FcγRIII engagement clears RhD<sup>+</sup> autologous red cells in human volunteers as efficiently as polyclonal anti-D antibodies. *Br J Haematol* 2008; **141**:109–19.
  - 26 Tandai M, Endo T, Sasaki S, Masuho Y, Kochibe N, Kobata A. Structural study of the sugar moieties of monoclonal antibodies secreted by human-mouse hybridoma. *Arch Biochem Biophys* 1991; **291**:339–48.
  - 27 Raju TS, Briggs JB, Borge SM, Jones AJS. Species-specific variation in glycosylation of IgG: evidence for the species-specific sialylation and branch-specific galactosylation and importance for engineering recombinant glycoprotein therapeutics. *Glycobiology* 2000; **10**:477–86.
  - 28 Medzhitov R, Janeway CA. Decoding the patterns of self and nonself by the innate immune system. *Science* 2002; **296**:298–300.
  - 29 Galili U. Interaction of the natural anti-Gal antibody with α-galactosyl epitopes: a major obstacle for xenotransplantation in humans. *Immunol Today* 1993; **14**:480–2.
  - 30 Sheeley DM, Merrill BM, Taylor LCE. Characterization of monoclonal antibody glycosylation: comparison of expression systems and identification of terminal α-linked galactose. *Anal Biochem* 1997; **247**:102–10.
  - 31 Chung CH, Mirakhur B, Chan E *et al.* Cetuximab-induced anaphylaxis and IgE specific for galactose-α-1,3-galactose. *New Engl J Med* 2008; **358**:1109–17.
  - 32 Kaneko Y, Nimmerjahn F, Ravetch JV. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science* 2006; **313**:670–3.
  - 33 Gaines AR. Disseminated intravascular coagulation associated with acute hemoglobinemia or hemoglobinuria following Rh<sub>0</sub> (D) immune globulin intravenous administration for immune thrombocytopenic purpura. *Blood* 2005; **106**:1532–7.
  - 34 Tarantino MD, Bussel JB, Cines DB *et al.* A closer look at intravascular hemolysis (IVH) following intravenous anti-D for immune thrombocytopenic purpura (ITP). *Blood* 2007; **109**:5527.